STUDIES ON THE PASTEUR EFFECT IN *ESCHERICHIA COLI* STRAIN B: ON THE FAILURE OF NITRATE TO REPLACE OXYGEN AS THE TERMINAL ELECTRON ACCEPTOR

by

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To my parents,

Michael and Stephanie Humniski.

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ABSTRACT

The existence of a Pasteur effect has been demonstrated in Escherichia coli strain B; with glucose acting as the substrate growth and CO_2 production were lower and lactate production higher under anaerobic conditions than under aerobic conditions. An attempt to provide more concrete evidence for the existence of the Pasteur effect through the inhibition of oxygen utilization by rotenone was not successful. The failure of rotenone to inhibit oxygen uptake is discussed.

Regardless of the presence of oxygen, whenever lactate and CO_2 were produced from glucose catabolism, their production was inhibited by nitrate. These results were confirmed with both growing and resting cell suspensions. In addition, nitrate inhibited the final cell density of both aerobically and anaerobically grown cells. Despite the inhibition of anaerobic lactate production, nitrate was not an effective terminal electron acceptor in the absence of oxygen.

The reduction of nitrate resulted in an accumulation of nitrite which was proven to inhibit both anaerobic and aerobic growth. In addition, nitrite inhibited anaerobic lactate production. It is suggested that nitrate fails to replace oxygen as the terminal electron acceptor because of the accumulation of the toxic nitrite ion.

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INTRODUCTION

INTRODUCTION

In the classical definition of the 'Pasteur effect', oxygen suppresses both the rate of carbohydrate breakdown as well as the formation of reduced end-products of anaerobic carbohydrate metabolism. It has been generally agreed that adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and inorganic phosphate (Pi) are the effectors of the regulation of the Pasteur effect (39). The allosteric enzyme phosphofructokinase (PFK) has been proposed as the principle regulator of glycolysis in yeast and therefore of the Pasteur effect (33). Its effectors have been demonstrated to be ATP, ADP, AMP, Pi, and citrate (24,33,40). Phosphofructokinase from E. coli (3) behaves similarly to that obtained from yeast.

The respiratory requirement for ADP and Pi in the governing of aerobic glycolysis has been linked to the nicotine adenine dinucleotide (NAD^+)-flavoprotein (F) region of the cytochrome system (8). The cytochrome system when coupled to oxidative phosphorylation produces energy in the form of ATP. Oxygen acts as the electron acceptor at the terminal end of this system and, in the presence of oxygen, 3 moles of ATP are produced for each mole of $NADH_2$ reduced.

The Pasteur effect has not been demonstrated in Escherichia coli. This organism does have a respiratory nitrate reductase. Dobrogosz (11) has demonstrated that nitrate

affects the end-product levels of anaerobic metabolism in E. coli and the nitrate reductase has been linked to energy production in an electron transport scheme; one ATP is produced for each $NADH_2$ reduced.

The role of oxygen as a regulator of the Pasteur effect has never been fully examined. Its sole function may be that of serving as a terminal electron acceptor or it may serve in other functions hitherto undisclosed. The purpose of this research has been firstly, to establish the presence of a Pasteur effect in E. coli and secondly, to determine whether molecular oxygen is mandatory for the operation of a Pasteur effect or whether it can be replaced by nitrate as an alternative terminal electron acceptor.

HISTORICAL

HISTORICAL

THE PASTEUR EFFECT

Since the original observation by Pasteur (7) that the presence of oxygen decreased the amount of alcohol produced and increased the amount and efficiency of growth in brewer's yeast, considerable work has been done in order to elucidate the Pasteur effect. Pasteur's description was essentially a qualitative concept of this naturally occurring phenomenon.

The Pasteur effect was left for others to express in quantitative terms. These measurements have been termed "oxidation quotients" and have been listed by Burke (7):

(a) The Myerhoff Oxidation Quotient

(b) The Myerhoff Quotient

(c) The Myerhoff Aerobic Oxidation Quotient

(d) The Kluyver Oxidation Quotient.

These quotients are arrived at by comparing end-products of metabolism, namely lactate, CO_2 or alcohol under aerobic as well as anaerobic conditions. The Myerhoff Oxidation Quotients and the Myerhoff Quotient have received general acceptance because their derivations are free from any hypothesis for the mechanism of the Pasteur effect. The Myerhoff Oxidation Quotient and the Kluyver Oxidation Quotient are based on such a hypothesis and their use has

been somewhat limited.

All organisms or tissues are not expected to show a Pasteur effect. The organism or tissue must be capable of carrying out metabolic processes both aerobically and anaerobically. In animal cells the Pasteur effect has been demonstrated in liver, sperm, bone marrow, brain and kidney as well as in benign and malignant tumors (7). In plant tissues it has been demonstrated in carrot roots, apples, potatoes, pea seeds, buckwheat seedlings, bananas and rhododendron leaves (5). The Pasteur effect has also been demonstrated in baker's yeast (26,43,44). Clearly the nature of the system under investigation regulates the choice of parameters which may be used to show a Pasteur effect in that system.

Wiken et al (51) have demonstrated the existence of a negative Pasteur effect with Saccharomyces carlsbergensis. In the presence of oxygen alcoholic fermentation was stimulated and in the absence of oxygen the fermentation was inhibited. This effect was obtained by treatment of cells with succinic acid-succinate buffer and by passage of very low oxygen concentrations into anaerobic cell suspensions prior to the addition of the substrate.

Since Myerhoff and Fiala (26) showed a Pasteur effect in sonically disrupted yeast this phenomenon has been demonstrated in a number of cell-free systems. Terner (48) has

shown a Pasteur effect in homogenates of rat and guinea pig mammary gland. Aisenberg et al (1,2) have shown that a Pasteur effect exists in reconstructed systems consisting of glycolizing brain extracts and respiring rat liver mitochondria. Racker and his associates (16,17) have continued this work with reconstructed systems replacing the glycolizing brain extracts mentioned above with the constituent enzymes of the Embden-Myerhoff pathway.

Many theories have been proposed to elucidate the mechanism of the Pasteur effect. Racker and Wu (54) have conveniently grouped these theories into three general categories:

(a) The Competition Theory

(b) The Structural Theory

(c) The Inhibition Theory

In the competition theory it has been proposed that in the presence of oxygen certain cofactors required by glycolysis have been removed by respiration. The rate of regeneration of ATP is dependent upon a competition between glycolysis and respiration for the precursors of ATP, namely Pi, AMP and ADP. Competition between respiration and glycolysis for Pi (20,53), AMP and Pi, (38,52), ADP and Pi (39,54) and AMP, ADP and Pi (8) has been suggested as the cause of the Pasteur effect.

In the structural theory it has been proposed (10) that

an expenditure of energy by respiration is responsible for keeping key enzymes of the cell inaccessible to their substrates. In aerobiosis the cell has become less permeable and consequently the substrate has become less accessible to the enzymes. Therefore aerobic conditions have lowered the rate of carbohydrate metabolism. Barker et al (4,5) have proposed a glycolytic structure that contains the enzymes of glycolysis. This structure has been proposed as being more permeable to ADP than to ATP under anaerobiosis.

In the inhibition theory it has been proposed that oxygen or an inhibitor formed in the presence of oxygen is responsible for the inhibition of glycolysis. Lipmann (22) proposed that oxygen reduced the rate of glycolysis by reversibly inactivating a sulfhydryl enzyme of glycolysis. The discovery by Terner (47) that 2,4-dinitrophenol eliminated the Pasteur effect without affecting respiration caused this theory to be revised. Racker (37) therefore suggested that an inhibitor of glucose utilization may have accumulated concomitant with oxidative phosphorylation.

Glyceraldehyde-3-phosphate dehydrogenase (54), hexokinase (9) and PFK (33,40) have all been proposed as enzymes regulating glycolysis. Phosphofructokinase has been suggested as rate-limiting in yeast (23,40,50), diaphragm (29) and brain (33) as well as in liver slices, Novikoff hepatomas and adenocarcinomas (52). Citrate (40) and excess ATP (24,33) have been reported as inhibitory to this allosteric enzyme.

The inhibition of ATP was relieved by ADP and Pi.

Further, Cereijo-Santalo and Wenner (8) have linked the respiratory requirement for ADP and Pi in the governing of aerobic glycolysis to the nicotine adenine dinucleotide (NAD^+)-flavoprotein (F) region of the cytochrome system. This view has been supported by Racker (38). Regardless of which theory of regulation of the Pasteur effect is espoused, the levels of high energy intermediates and products of phosphorylation play a key role as governors of this reaction.

TERMINAL ELECTRON ACCEPTORS

A terminal electron acceptor is the ultimate electron acceptor in a series of reactions involving electron flow; the efficiency of the terminal electron acceptor is determined by the net energy produced. Because of its high affinity for electrons and because its reduction does not require an expenditure of biological energy, oxygen is the most efficient and most widely used terminal electron acceptor; the product of the reaction is H_2O.

But oxygen is not the only terminal electron acceptor participating in electron transfer mechanisms. The genus Desulfovibrio for example, includes sulfate-reducing bacteria (35). This specialized group of bacteria use sulfate as the terminal electron acceptor under anaerobic conditions. Peck (34) has illustrated the reduction of

sulfate to sulfide with molecular hydrogen in Desulfovibrio desulfuricans:

$$SO_4^{--} + 4\ H_2 \longrightarrow S^{--} + 4\ H_2O$$

The electrons can be derived from other donors such as acetate. Sulfate, however, is not an efficient terminal electron acceptor, since its reduction requires an expenditure of energy.

A number of terminal electron acceptors have been used with artificial systems. Quastel (36) and Dolin (12) have summarized these artificial electron acceptors and their uses. Methylene blue has been used for the study of muscle dehydrogenases, bacterial enzyme reactions and carrier-linked reactions. Ferricyanide has been used to estimate glutathione and NAD^+ as well as to study various dehydrogenase systems. Manganese dioxide, 2,6-dichlorophenol and the tetrazolium salts have also been used as artificial electron acceptors.

ESCHERICHIA COLI AND NITRATE RESPIRATION

The Pasteur effect has not been demonstrated in E.coli. Phosphofructokinase from this organism however has been found (3) to be similar to the PFK found in yeast. Inorganic phosphate and ADP stimulated while excess ATP inhibited the enzyme. Dobrogosz (11) reported that nitrate altered end-product patterns of anaerobic growth in the presence of

nitrate became similar to aerobic end-product patterns. Nitrate however did not completely change the end-product patterns of anaerobic growth. The data he presented were semi-quantitative.

Spangler and Gilmour (42) have presented quantitative data on the effect of nitrate on glucose and gluconate utilization as well as CO_2 production by Pseudomonas stutzeri. These parameters were measured by radioactive tracer techniques with both aerobic and anaerobic cell suspensions. The accuracy afforded by the technique used gives a more accurate picture of the effect of nitrate on the parameters tested.

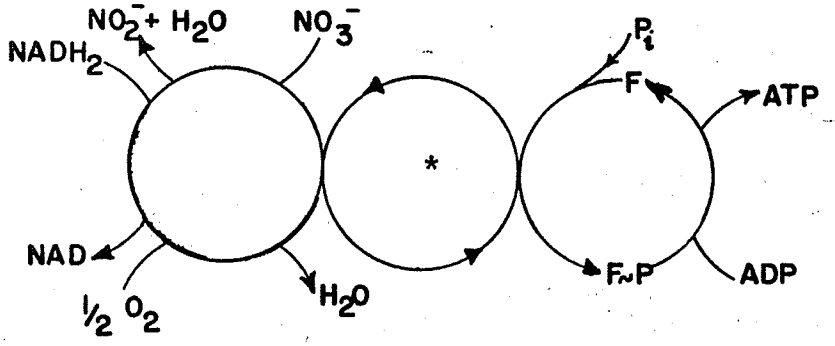
Many organisms have the ability to reduce nitrate. When the reduction products of nitrate serve to form nitrogenous cell components the mechanism is termed nitrate assimilation. Nitrate respiration as proposed by Sato (41) represents the utilization of nitrate or some of its reduction products as terminal electron acceptors in place of oxygen.

Hadjipetrou and Stouthamer have claimed that nitrate reduction coupled with anaerobic glucose metabolism has been inferred to yield 3 moles of ATP per mole of nitrate reduced in Aerobacter aerogenes (18). The scheme for nitrate reduction in E. coli presented later in this section however infers the production of 1 mole of ATP per mole of nitrate reduced. Aerobic glucose catabolism proceeding through

the Embden-Myerhoff pathway and the Krebs cycle would produce 38 moles of ATP per mole of glucose. Anaerobic nitrate respiration coupled to glucose catabolism could never attain this efficiency because the reduction of nitrate is energy requiring (27). But it is not unreasonable to expect nitrate to increase the efficiency of energy production through anaerobic glucose catabolism.

Nitrate reductase has been shown by various workers (19,30,32,41,45,46) to exist in E. coli. Nitrite reductase has also been demonstrated in this organism by Lazzarini and Atkinson (21). Ota et al (32) however have shown that E. coli strain K-12 does not utilize nitrite as a terminal electron acceptor.

The following scheme has been suggested by Ota (31) as the sequence of electron transport in E. coli.



In the scheme above, * signifies unknown intermediate(s) blocked by dinitrophenol.

Pathways for electron transport in other systems have been published and these may be found in the reviews of Fewson (14) and Nason (27).

It is generally accepted that the levels of high energy intermediates and products of phosphorylation play the key role as governors of the Pasteur effect. Theories proposed to elucidate the mechanism of the Pasteur effect however fail to assign a truly definitive role to oxygen. It is clear that oxygen is the most efficient electron acceptor but an effective replacement of oxygen by nitrate might clarify the role of oxygen in the mechanism of the Pasteur effect.

MATERIALS AND METHODS

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CULTURE

The organism used for this study was a laboratory culture of *Escherichia coli* strain B.

GROWTH MEDIUM

The organism was grown in a glucose mineral salts medium composed of:

Glucose	5.0 gm
KH_2PO_4	10.5 gm
$(NH_4)_2SO_4$	1.0 gm
$(NH_4)6Mo_7O_{24}.4H_2O$	6.4×10^{-5} gm
$MgSO_4$	0.05 gm
Distilled H_2O	1000 ml

To prevent precipitation of magnesium phosphate, the $MgSO_4$ was added only after the other salts had dissolved. The mineral salts medium was routinely prepared at 10 times concentration and asceptically added to a separately autoclaved glucose solution in distilled water.

GLUCOSE DETERMINATION

Glucose was measured quantitatively by the procedure of Folin and Malmros (15). The following reagents were required: 0.4% $K_3Fe_2(CN)_6$; carbonate-cyanide solution; iron solution. The carbonate-cyanide solution and the iron solution were prepared as follows:

Carbonate-Cyanide Solution: To 50 ml of 10% Na_2CO_3 solution were added 5 ml of a 1% NaCN solution and the total volume was adjusted to 500 ml with distilled water.

Iron Solution: A gauze bag containing 20 gm of gum ghatti was soaked overnight in 1 litre of water. After soaking, the bag was removed and 5 gm of $Fe_2(SO_4)_3$, 75 ml of 85% H_3PO_4 and 15 ml of 1% $KMnO_4$ were added to the solution. The solution was stored for several days prior to use.

To a sample containing glucose, 3 ml of water, 2 ml of $K_3Fe_2(CN)_6$ and 1 ml of the carbonate-cyanide solution were added. After 10 minutes immersion in a boiling water bath, the sample tubes were cooled by plunging into ice for 1 to 3 minutes. Then 5 ml of the iron solution was added and the volume was adjusted to 25 ml with distilled water. The sample tubes were allowed to stand for 20 minutes at room temperature to insure maximum color development. All samples, including a reagent blank as a control, were measured in a Klett-Summerson photoelectric colorimeter equipped with a KS-54 filter. Stoichiometry was observed up to 100 µgm glucose. The glucose concentration in samples undergoing analysis was always determined from freshly prepared standard curves.

NITRATE DETERMINATION

Nitrate was determined by a modification of the method

of Montgomery and Dymock (25). The reagents employed for the test were prepared as follows:

(a) Sulphuric Acid 80.5-83.33% (w/w): 455 ml of reagent grade sulphuric acid was mixed with 171 ml of nitrate-free water.

(b) NH_4Cl Solution: 24 gm of NH_4Cl were dissolved in 100 ml of distilled water.

(c) 2,6-Dimethyl phenol: 0.122 gm of 2,6-dimethyl phenol were dissolved in 50 ml of reagent grade concentrated acetic acid and the volume was adjusted to 100 ml with the NH_4Cl solution.

(d) Sulphamic Acid Squares: Whatman #5 filter paper was cut into 1 cm squares and were then immersed in a solution of 50% sulphamic acid. The squares were allowed to dry and stored in a stoppered bottle at 4°C.

To 1.6 ml of the H_2SO_4 solution (cooled to 0-10°C) were added 0.1 ml of sample and 0.1 ml of 2,6-dimethyl phenol reagent. After 5 minutes, 3 ml of distilled water were added and the mixture was allowed to stand for 15 minutes at room temperature. The optical density was measured at 304 mμ in a Unicam SP-700 spectrophotometer using a reagent blank as a control. The optical density remained unchanged for 2 hours at room temperature or for 2 days at 4°C.

Nitrite when present in a sample was removed by the addition of a sulphamic acid square. After 5 minutes

reaction, the square was removed. A single sulphamic acid square will destroy 0.1 mgm of nitrite-nitrogen.

NITRITE DETERMINATION

Nitrite was determined by the sulfanilic acid procedure of Bratton et al.(6). The reagents required were 0.02% N-(1-naphthyl)-ethylenediamine dihydrochloride in distilled water and 1% sulfanilic acid in 20% HCl.

Aliquots of each sample were adjusted to 6 ml with deionized water, and 0.5 ml of sulfanilic acid and 0.5 ml of N-(1-naphthyl)-ethylenediamine dihydrochloride were added. The volume was adjusted to 20 ml with deionized water and the mixture was allowed to stand for 20 minutes at room temperature to permit maximum color development. A reagent blank was used as a control and the color intensity was measured in a Klett-Summerson photoelectric colorimeter using the KS-54 filter.

A standard curve was prepared by the above method using known amounts of nitrite. Under the conditions employed, 1 mμmole of nitrite corresponded to 1.4 Klett units.

LACTATE DETERMINATION

Lactate was determined by the method of Eegriwe (13). The reagents required were 4% aqueous $CuSO_4$, reagent grade sulphuric acid and 1% p-phenylphenol dissolved in 0.08 N NaOH. The p-phenylphenol was stored in a brown bottle at $4^{o}C$.

To 1 ml of the test solution were added 1 drop of 4% $CuSO_4$ and 6 ml of concentrated sulphuric acid. The mixture was allowed to stand for 5 minutes at room temperature and then cooled to below 20°C by immersion in a cold water bath. One drop of p-phenylphenol was added, and the solution was thoroughly mixed and allowed to stand for 8 hours at room temperature. The optical density was read in a Klett-Summerson photoelectric colorimeter using a KS-54 filter. Reagent blanks served as controls. A reading of 2.5 Klett units corresponded to 1 mμmole of lactate.

Lactate was recovered from the samples by ether extraction. Figure 1 shows the extraction apparatus used. A 6 ml aliquot of the sample was placed in the ether extraction sample reservoir, and then saturated with NaCl and acidified with 2 drops concentrated reagent grade sulphuric acid to increase the efficiency of extraction. A low heat was applied to the collection flask with a Snelling-Fisher Hotspotter. The ether vaporized and condensed at the top in the cold water condensor. The condensed ether which accumulated in the funnel, being immiscible with and less dense than water, was forced upward through the sample mixture carrying with it the lactate in the sample. The layer of ether escaped through the aperture in the side of the sample tube and dripped down into the collection flask. Lactate accumulated in the collection flask due to its high boiling point.

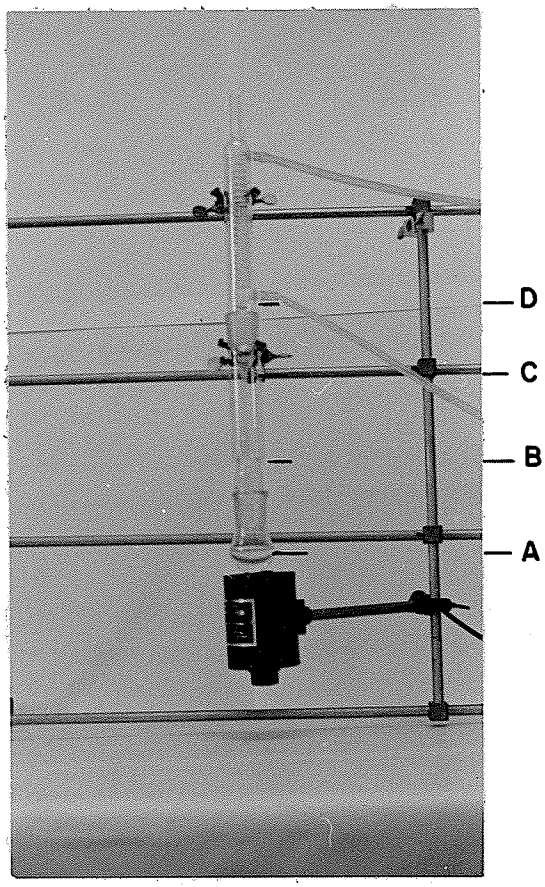
FIGURE 1. The lactate extraction apparatus.

A. Collection flask

B. Sample flask

C. Funnel

D. Condenser


D
C
B
A

After 24 hours of continuous extraction the apparatus was dismantled and the ether was washed out of the collection flask with distilled water. The ether was removed by evaporation under vacuum and the residue was taken up in water and was then adjusted to 12 ml. With 24 hours of continuous extraction, by this method, 89% recovery of lactate, provided as lithium lactate, was achieved.

CARBON DIOXIDE DETERMINATION

Carbon dioxide was measured by direct titration after conversion to $BaCO_3$ (28). The CO_2 liberated during growth was collected in CO_2 traps containing KOH as the absorbing agent, (see Fig.2). After the completion of growth, the KOH was washed out of the CO_2 traps with CO_2-free distilled water. The contents of the trap were adjusted to 100 ml with carbon dioxide-free distilled water. After mixing, the KOH solution was divided into two 50 ml portions. To each portion, 5 ml of saturated $BaCl_2$ and 2 drops of 0.5% phenolphthalein in 50% ethanol were added. The excess base was neutralized with HCl to the phenolphthalein end-point. One drop of 0.5% bromphenol blue was then added to the sample and the $BaCO_3$ was then dissociated with standardized HCl. In all trials a correction was made for endogenous CO_2 production using the same procedures.

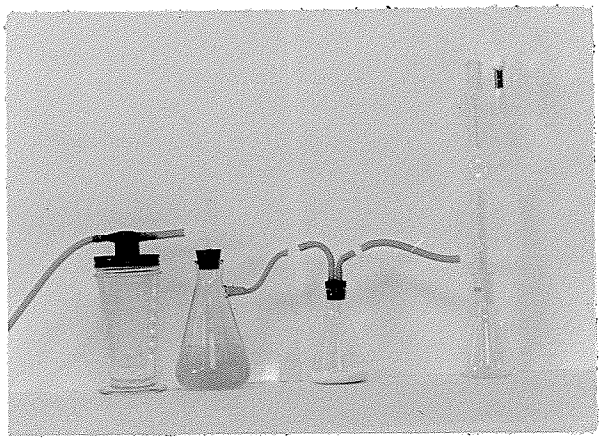
FIGURE 2. The aerating train used in aerobic experiments with growing and resting cells.

A. Fisher-Mulligan Gas Washer

B. Distilled Water Trap

C. Growth Flask

D. CO_2 Bubble Tower



A B C D

GROWING CELL EXPERIMENTS

Experiments were conducted to test the effects of nitrate and nitrite on aerobically and anaerobically growing cells. Nephelometer flasks fitted with 2-holed rubber stoppers at the neck were used for aerobic growth. For anaerobic growth studies, Nephelometer flasks were fitted with ground glass stopcocks at the top and male ports at the side.

The flasks containing 46 ml of mineral salts medium and various concentrations of nitrate or nitrite were autoclaved separately and glucose was added aesceptically to a final concentration of 0.5%. From each flask a 1 ml sample was removed asceptically and stored at -20° for future tests.

Inocula for these experiments consisted of a 24 hour culture grown on the same medium under aerobic conditions. The cell density of the inoculum was adjusted with sterile water to 200 Klett units using a KS-54 filter and each flask received 0.5 ml of inoculum.

At this point, the cotton plugs in the ports of the anaerobic culture-flasks were replaced with sterile serum caps. Sterile carbon dioxide-free nitrogen was introduced by means of a sterile needle inserted through the serum cap. After 5 minutes of bubbling, the stopcock was closed and the needle was removed.

Aerobic growth flasks were set up in aerating trains as shown in Figure 2. Air was passed through concentrated

NaOH in a Fisher-Mulligan gas washer to remove atmospheric CO_2 and then through a distilled water trap. The distilled water trap served to replace moisture and, as a safety feature, to prevent any NaOH from reaching the cells. The CO_2-free air served as a carrier gas to sweep the CO_2 produced by fermentation into the KOH trap which contained approximately 25 ml of KOH. Aeration by the above method was carried out until the stationary phase of growth was reached, usually about 40 hours.

Both aerobic and anaerobic cultures were grown at room temperature on a Northcott rotary shaker set at 120 cycles per minute at an eccentricity of 1.4 cm. Growth was measured by determining optical density on a Klett-Summerson photoelectric colorimeter using a KS-54 filter at suitable time intervals.

After growth had reached the stationary phase (usually approximately 40 hours), 0.1 ml of 0.2 N H_2SO_4 was added to each flask to stop the fermentation. The aerobic culture flasks were disconnected and the bubble towers were sealed off. Carbon dioxide-free nitrogen gas was forced under pressure through each of the anaerobic culture flasks into a KOH bubble tower for about 5 minutes. The pH of the contents of each flask was adjusted to neutrality and the total volume was measured. Cells were removed by centrifugation and the fermentation liquor was stored at -20°C until required. The levels of nitrate, nitrite, glucose

and lactate in the samples taken before and after growth as well as the amount of CO_2 produced were determined.

Nitrite reductase may have been inhibited by the ammonium ion present in the medium, resulting in the accumulation of inhibitory nitrite ions. To exclude this possibility, the $(NH_4)_2SO_4$ of the mineral salts medium was replaced by 0.2% D,L-glutamic acid, as the nitrogen source. A 0.1% solution of K_2SO_4 was also added with the glutamic acid solution. The concentration of nitrate, as the potential terminal electron acceptor, was varied and growth was the only parameter measured.

RESTING CELL EXPERIMENTS

The effects of nitrate and nitrite upon aerobic and anaerobic suspensions of resting cells were tested. Cells were grown at room temperature in 15 litre carboys containing 12 litres of the standard glucose-mineral salts medium supplemented with 0.4% KNO_3. The inoculum consisted of 500 ml of a 24 hour culture of the cells. Aeration was achieved by passing air, sterilized by filtration through sterile cotton filters, into the medium where it was dispersed by means of fritted glass spargers. Sterile nitrogen was bubbled in the same manner through the medium to achieve anaerobic conditions.

After 12 hours of growth the cells were centrifuged in

a steam-driven Sharples Super centrifuge at a speed of 20,000 revolutions per minute (20 pounds per square inch), and then washed twice with 0.1 M potassium phosphate buffer pH 7.0. Cells were resuspended in the same buffer and the cell density was adjusted so that a 1:10 dilution of the suspension gave a reading of 500 Klett units.

Aerobic culture-flasks containing 40 ml of cell suspension were connected to the aerating train (Fig.2). After 10 minutes of equilibration achieved by passing CO_2-free air through the system, 200 µmoles of glucose and various concentrations of nitrate or nitrite were added to each flask by means of a syringe inserted through the rubber stopper. Flasks were shaken on a Northcott rotary shaker at 120 cycles per minute at an eccentricity of 1.4 cm. After 2 hours of respiration at room temperature, 3 ml of 2 N H_2SO_4 were used to stop enzyme activity and to drive dissolved CO_2 from solution.

Carbon dioxide-free nitrogen instead of air was used as the carrier gas to remove the CO_2 produced by the cell suspension under anaerobic conditions. The same procedures and analytical tests were carried out as previous described with growing cells.

MANOMETRIC EXPERIMENTS

According to Cereijo-Santalo and Wenner (8) rotenone is

able to inhibit glucose oxidation. Measurements of oxygen uptake and lactate production provided a simple demonstration of the Pasteur effect.

Manometric experiments were carried out in a Bronwill Warburg respirometer at 30°C with air as the gas phase using standard manometric techniques (49). Cells were grown on the glucose-mineral salts medium in two-litre Fernbach flasks containing 1500 ml of medium and inoculum. Cells were grown for 12 hours at room temperature on a Northcott rotary shaker set at 150 cycles per minute at an eccentricity of 1.4 cm, and then harvested in a Sorvall refrigerated centrifuge, model RC-2, using the GSA rotor at 10,000 times gravity. Cells were washed twice in 0.1 M potassium phosphate buffer pH 7.0 and then resuspended in the same buffer so that a 1:10 dilution gave a reading of 240 Klett units.

Each Warburg flask in the ensuing experiments contained: 0.2 ml of 20% KOH and a small fluted filter paper in the centre well; 60 umoles of glucose in the side arm; 0.5 ml (17 mgm wet weight) of the standardized cell suspension and 125 umoles of potassium phosphate buffer pH 7.0 in the main compartment of the flask. When required, 0.01 ml of rotenone (Sigma Chemical Co.) dissolved in 95% ethyl alcohol was added to the main compartment of the flask, giving a final concentration of 0.7×10^{-6} M rotenone. In flasks with no rotenone, the same volume of 95% ethanol was substituted

in order to compensate for the physiological effect of alcohol.

In an attempt to increase the permeability of the cells to rotenone, 0.2 ml of ethylene diamine tetraacetic acid (EDTA) pH 7.0, was added in various concentrations to the second side arm in some experiments. In addition, cells were also treated with various dilutions of Tween 80. The Tween 80 was diluted in 0.1 M potassium phosphate buffer pH 7.0 and 9 ml of this dilution was added to 1 ml of standardized cell suspension at 4°C. After 24 hours, Tween 80 was removed from the cells by centrifugation. Cells were washed once with 0.1 M potassium phosphate buffer pH 7.0, were suspended in the same buffer and standardized to 200 Klett units as previously described. Oxygen uptake was then measured.

RESULTS

RESULTS

GROWTH OF E. COLI UNDER AEROBIC AND ANAEROBIC CONDITIONS

Escherichia coli strain B grew well in the glucose-mineral salts medium under both aerobic and anaerobic conditions but growth aerobically, as expected, was significantly greater than that achieved anaerobically. Figure 3 shows that increasing the glucose concentration of the growth medium beyond 0.5% did not increase the amount of aerobic growth achieved and this substrate concentration was routinely employed throughout. The effect of substrate concentration upon the final cell density achieved under anaerobic conditions was not tested.

THE EFFECT OF NITRATE ON GLUCOSE METABOLISM BY GROWING CELLS

Table I shows the effect of nitrate on cell density, on lactate, CO_2 and nitrite production as well as on glucose and nitrate utilization by growing cells under anaerobic conditions. The addition of nitrate to the glucose-mineral salts medium reduced growth but the degree of nitrate reduction decreased as the nitrate concentration was increased. Figure 4a shows that the highest concentration of nitrate used (15 mmoles/50 ml) not only reduced the final cell density achieved but also increased the length of the lag phase of growth. Lactate was found to be the major cleavage-product of anaerobic glucose catabolism with

FIGURE 3. Effect of various concentrations of glucose upon aerobic growth of E.coli strain B. Glucose concentrations are expressed as percentage glucose supplied to the medium.

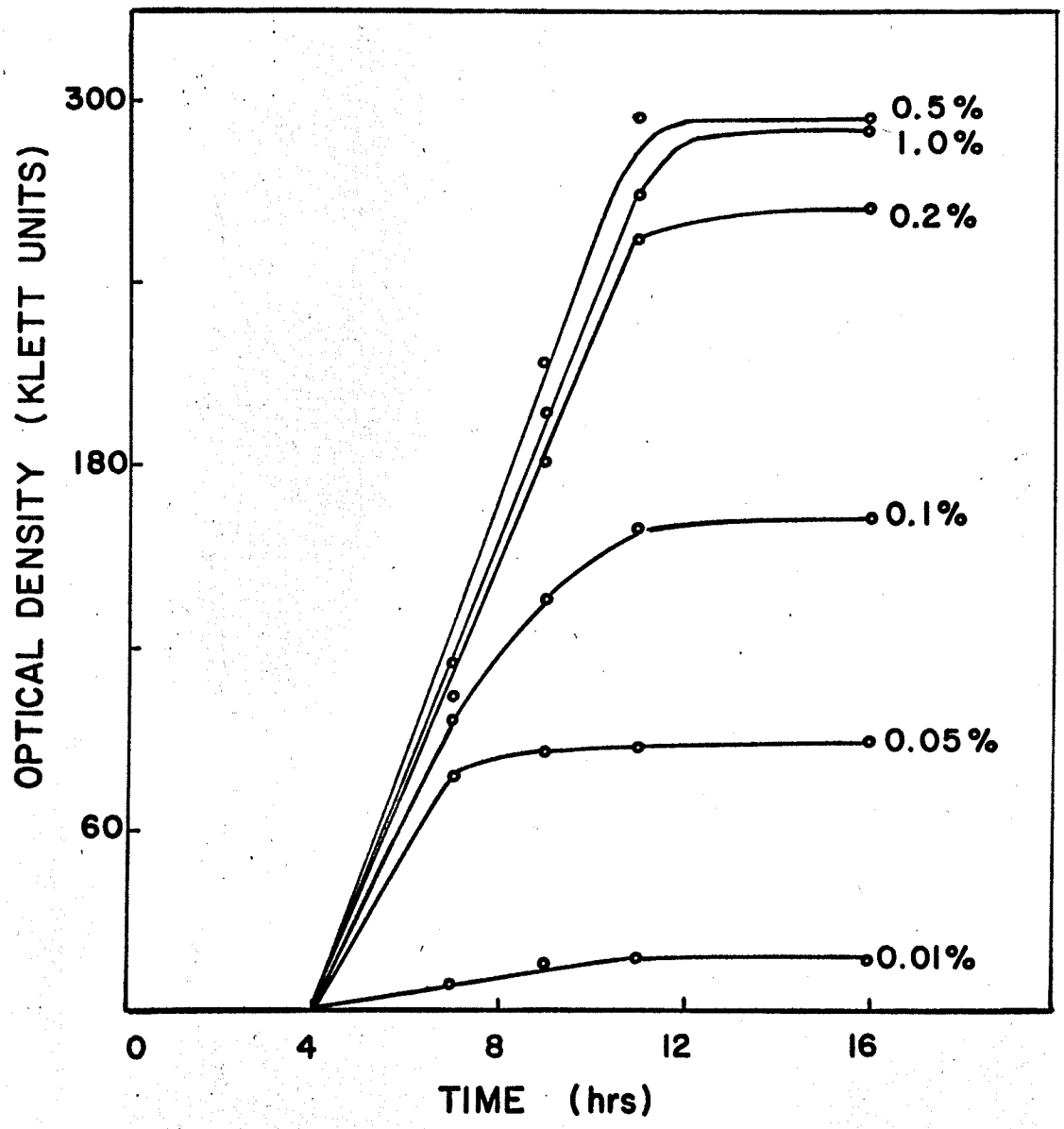
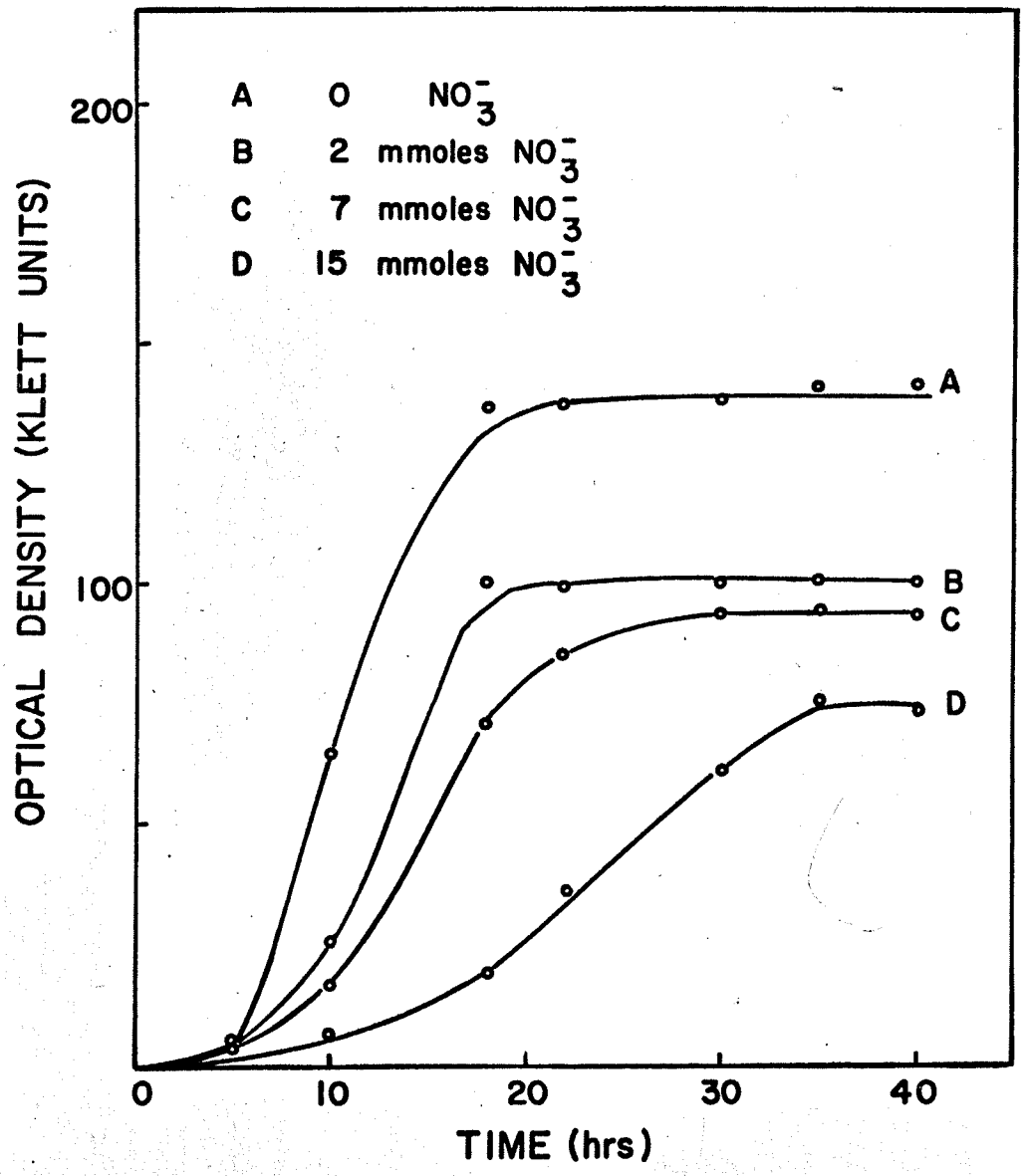

300
180
60
OPTICAL DENSITY (KLETT UNITS)
0.5%
1.0%
0.2%
0.1%
0.05%
0.01%
0
4
8
12
16
TIME (hrs)

TABLE I. The effect of nitrate on glucose metabolism by growing cell suspensions of E. coli under anaerobic conditions.

NO_3^- supplied (mmoles/50 ml)	Glucose supplied (mmoles)	Growth (Klett units per mmole glucose utilized)	Glucose utilized (mmoles)	CO_2 prod. (mmoles per mmoles glucose utilized)	Lactate prod.(mmoles per mmoles glucose utilized)	% carbon recovered as lactate and CO_2	NO_3^- utilized (mmoles per mmole glucose utilized)	NO_2^- prod. (mmoles per mmole glucose utilized)
0	1.40	104	1.36	0.01	0.90	44.8	-	-
2	1.40	72	1.40	0.09	0.65	33.8	0.75	0.72
7	1.40	73	1.27	0.03	0.68	34.4	0.60	0.57
15	1.40	61	1.21	0.05	0.78	37.4	0.23	0.25

FIGURE 4a. Effect of nitrate on the growth of *E. coli* strain B under anaerobic conditions. Concentrations are expressed as mmoles of NO_3^- supplied as KNO_3 to the medium.


A 0 NO_3^-
B 2 mmoles NO_3^-
C 7 mmoles NO_3^-
D 15 mmoles NO_3^-
OPTICAL DENSITY (KLETT UNITS)
200
100
0
10
20
30
40
TIME (hrs)
A
B
C
D

CO_2 produced in lesser amounts. Table I also shows that nitrate had little or no effect on glucose utilization. Yet lactate production was inhibited 14 to 28% by the presence of nitrate. The alternate fate of the glucose diverted by nitrate from lactate and CO_2 formation was not determined.

Figure 4b shows that nitrate inhibited aerobic growth. Table II shows that CO_2 was the major cleavage-product found in aerobic glucose catabolism. While only low amounts of residual glucose were evident, nitrate inhibited CO_2 production appreciably. Lactate production was very low in the absence of nitrate; the addition of nitrate to the growth medium did not appear to influence the amount of lactate formed. The quantities of nitrate utilized and nitrite produced were also very small.

A comparison of Tables I and II shows that nitrate is reduced only under anaerobic conditions. The pattern of glucose catabolism shifted from lactate production under anaerobic conditions to CO_2 production under aerobic conditions. A comparison of Figures 4a and 4b shows that nitrate was much more inhibitory to growth under anaerobic conditions than under aerobic conditions.

THE EFFECT OF NITRATE ON GLUCOSE METABOLISM BY RESTING CELL SUSPENSIONS

Table III shows the effect of nitrate on lactate, CO_2

FIGURE 4b. Effects of nitrate on the growth of *E.coli* strain B under aerobic conditions. Concentrations are expressed as mmoles of NO_3^- supplied as KNO_3 to the medium.

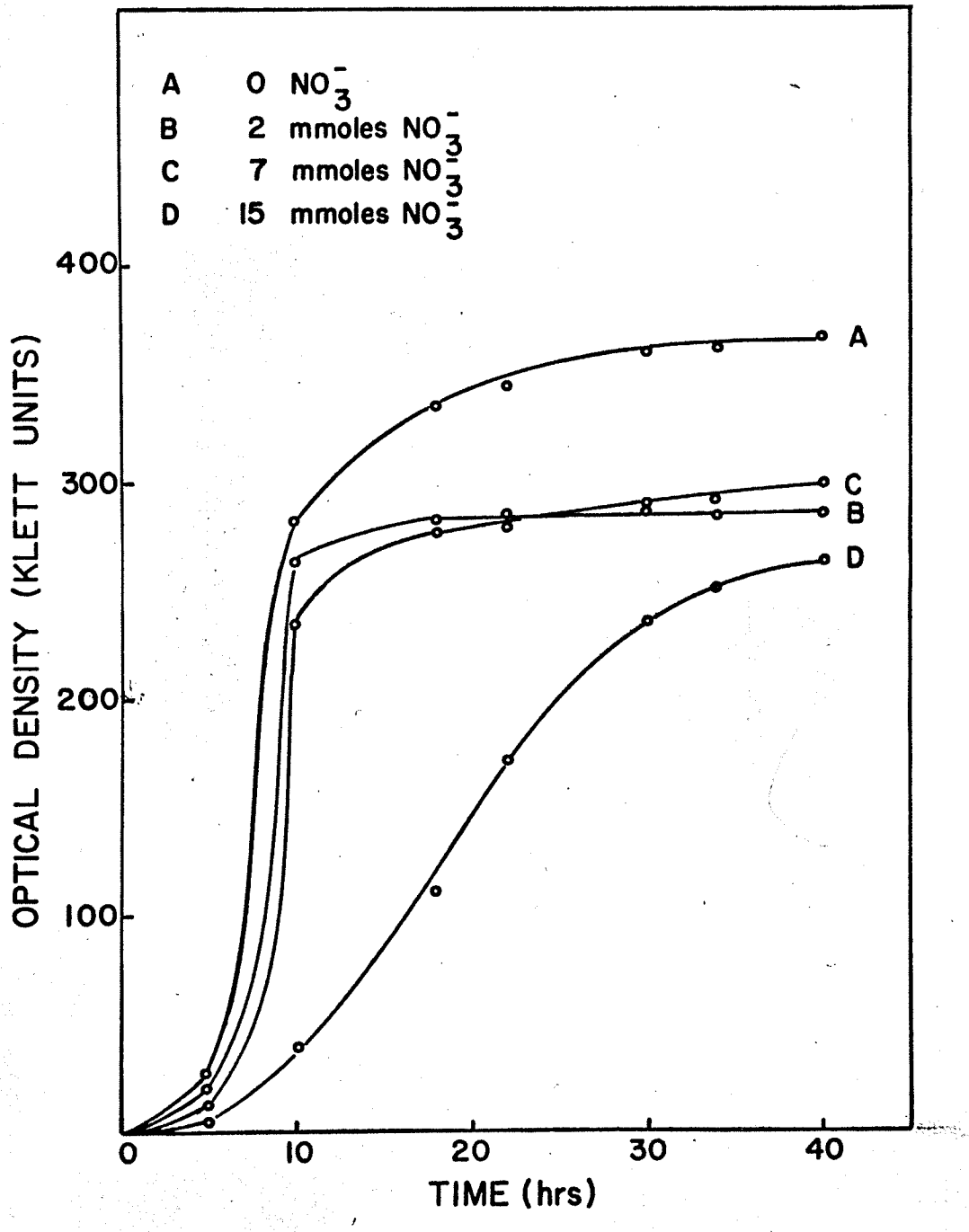

A 0 NO_3^-
B 2 mmoles NO_3^-
C 7 mmoles NO_3^-
D 15 mmoles NO_3^-
OPTICAL DENSITY (KLETT UNITS)
400
300
200
100
0
10
20
30
40
TIME (hrs)
A
C
B
D

TABLE II. The effect of nitrate on glucose metabolism by growing cell suspensions of E. coli under aerobic conditions.

NO_3^- supplied (mmoles/50 ml)	Glucose supplied (mmoles)	Growth (Klett units per mmole glucose utilized	Glucose utilized (mmoles)	CO_2 prod. (mmoles per mmoles glucose utilized	Lactate prod. (mmoles per mmoles glucose utilized)	% carbon recovered as lactate and CO_2	NO_3^- utilized (mmoles per mmoles glucose utilized)	NO_2^- prod. (mmoles per mmole glucose utilized)
0	1.40	267	1.38	3.89	<0.01	64.9	-	-
2	1.40	206	1.39	3.60	<0.01	60.1	<0.01	<0.01
7	1.40	216	1.39	3.43	<0.01	57.6	<0.01	<0.01
15	1.40	174	1.40	2.73	<0.01	45.9	<0.01	<0.01

TABLE III. The effect of nitrate on glucose metabolism by resting cell suspensions of E. coli under anaerobic conditions.

NO_3^- supplied (µmoles/50 ml)	Glucose supplied (µmoles)	Glucose utilized (µmoles)	CO_2 produced (µmoles per µmole glucose utilized)	Lactate prod. (µmoles per µmole glucose utilized)	NO_3^- utilized (µmoles per µmole glucose utilized)	NO_2^- produced (µmoles per µmole glucose utilized)
0	200	170	N.D.	5.14	-	-
1	200	193	N.D.	<0.01	0.86	0.82
5	200	193	N.D.	<0.01	0.45	0.38
15	200	191	N.D.	<0.01	0.60	0.78
25	200	193	N.D.	<0.01	0.60	0.71

N.D. - None detected.

and nitrite production as well as on glucose and nitrate utilization by resting cell suspensions under anaerobic conditions. Nitrate slightly stimulated the amount of glucose utilized or interfered to a slight extent with the glucose assay method. No carbon dioxide production could be detected. Nitrate completely inhibited lactate production. While the glucose underwent catabolic changes, the fate of the carbon supplied as glucose was not ascertained and a detailed carbon balance is not within the scope of this investigation. The amounts of nitrite produced were approximately the same as the amounts of nitrate metabolized. No clear correlation was evident between the amount of nitrate utilized and the amount of nitrate added. The results clearly show that nitrate cannot replace oxygen as a terminal electron acceptor under anaerobic growth conditions.

Table IV shows that with resting cell suspensions nitrate stimulated CO_2 production under aerobic conditions but only at low nitrate concentrations while lactate production was inhibited. The data did not reveal a relationship between the amount of nitrate utilized and the amount of nitrate supplied. The correlation between nitrite production and nitrate utilization was not as evident as with aerobically growing cells (see Table II).

Experiments on resting cell suspensions under aerobic conditions were carried out as controls to determine the effect of nitrate upon glucose catabolism by anaerobic

TABLE IV. The effect of nitrate on glucose metabolism by resting cell suspensions of E. coli under aerobic conditions.

NO_3^- supplied (µmoles/50 ml)	Glucose supplied (µmoles)	Glucose utilized (µmoles)	CO_2 produced (µmoles per µmole glucose utilized)	Lactate prod. (µmoles per µmole glucose utilized	NO_3^- utilized (µmoles per µmole glucose utilized)	NO_2^- produced (µmoles per µmole glucose utilized)
0	200	170	1.57	1.10	-	-
1	200	163	2.20	0.12	0.17	0.13
5	200	165	2.12	0.11	0.22	0.22
15	200	161	2.30	0.21	1.20	0.27
25	200	153	1.66	0.14	0.15	0.18

resting cell suspensions. A comparison of Tables III and IV shows that nitrate is reduced much more effectively (four fold) under anaerobic conditions. The production of lactate was lower and that of CO_2 higher under aerobic conditions than under anaerobic conditions.

THE EFFECT OF NITRITE ON GLUCOSE METABOLISM BY GROWING CELLS

Figure 5a shows the effect of nitrite on the growth of E.coli strain B under anaerobic conditions. Nitrite was supplied to the medium at concentrations ranging from 0.1 to 1.3 mmoles/50 ml; nitrate reduction had previously produced nitrite concentrations from 0.12 to 0.8 mmoles/ 50 ml with cells growing under anaerobic conditions. At the lowest concentration of nitrite tested growth was inhibited 46%. At higher levels of nitrite (0.3 and 0.5 mmoles/50 ml) only a small amount of growth occurred.

The same amounts of nitrite were supplied to the medium to determine the effect of nitrite on growth under aerobic conditions even though aerobic reduction of nitrate produced only traces of nitrite. Figure 5b shows that nitrite also inhibited growth under aerobic conditions although aerobic inhibition was much less marked than anaerobic inhibition by nitrite as in Figure 5a. At the lowest concentration of nitrite aerobic growth was inhibited 5%, while at the highest concentration of nitrite growth was

FIGURE 5a. Effect of nitrite on growth of *E.coli* strain B under anaerobic conditions. Concentrations are expressed as mmoles of NO_2^- supplied as KNO_2 to the medium.

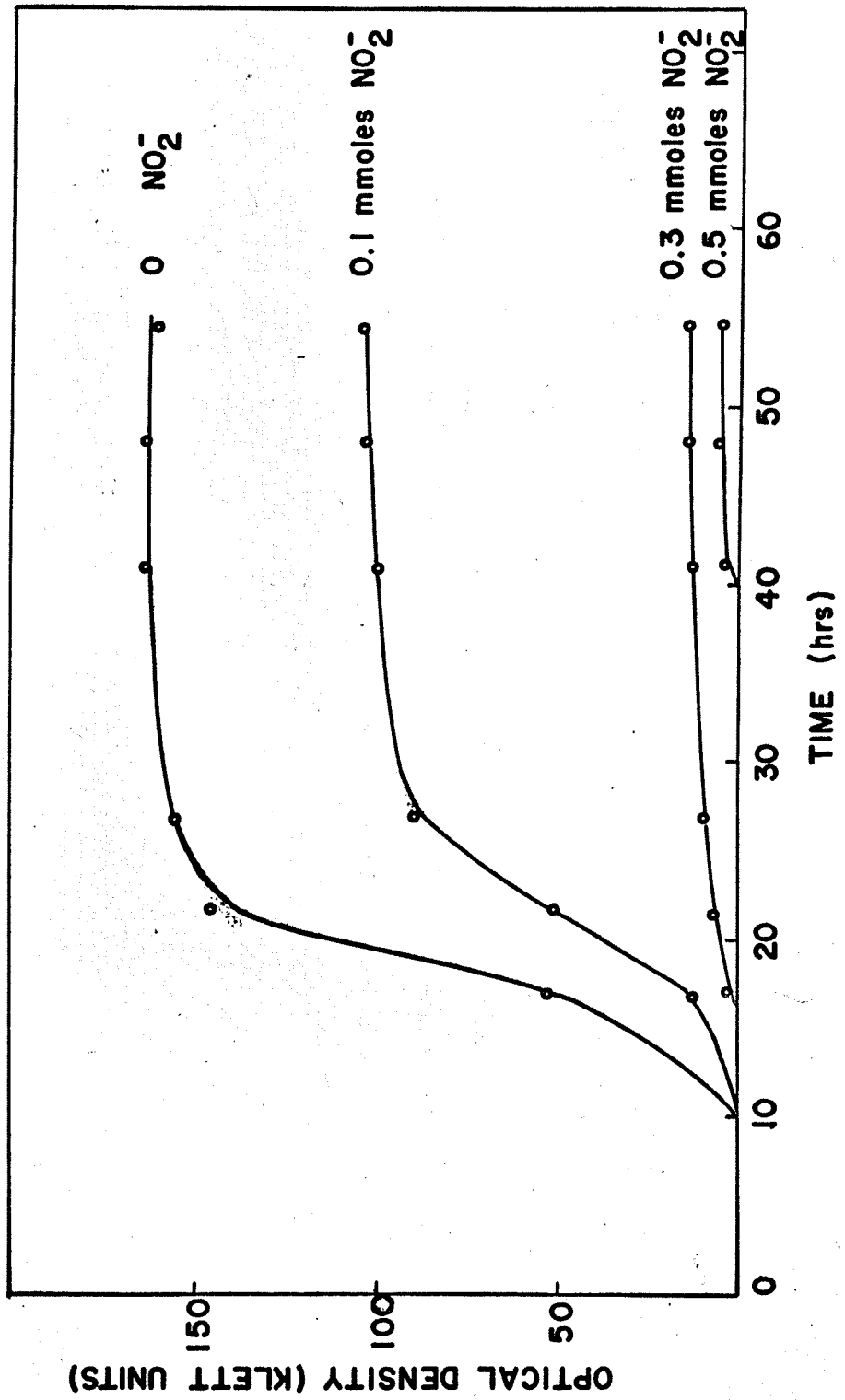
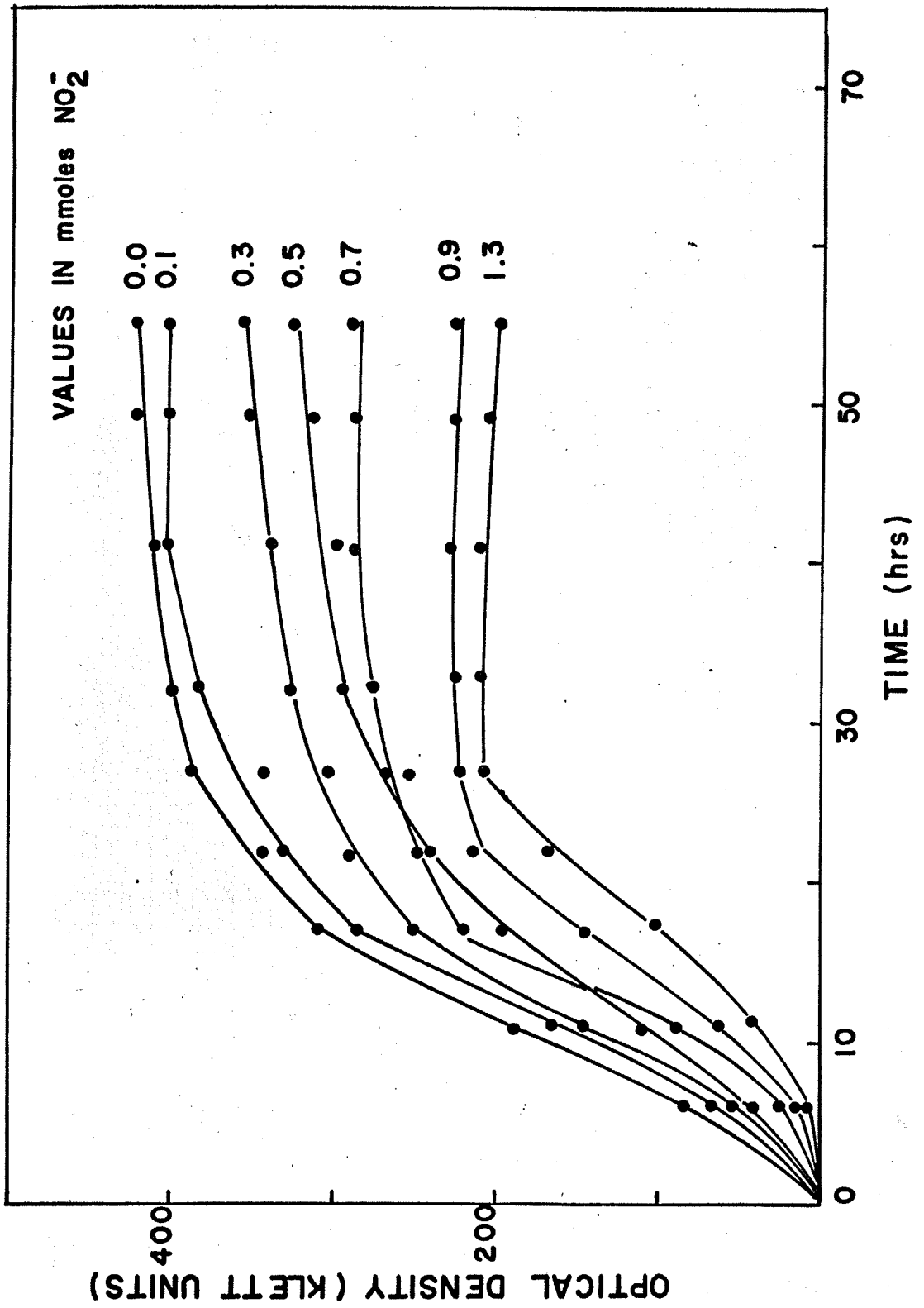

0 NO_2^-
0.1 mmoles NO_2^-
0.3 mmoles NO_2^-
0.5 mmoles NO_2^-
OPTICAL DENSITY (KLETT UNITS)
150
100
50
0
10
20
30
40
50
60
TIME (hrs)

FIGURE 5b. Effect of nitrite on growth of E.coli strain B under aerobic conditions. Concentrations are expressed as mmoles of NO_2^- supplied as KNO_2 to the medium.


VALUES IN mmoles NO_2^-
0.0
0.1
0.3
0.5
0.7
0.9
1.3
OPTICAL DENSITY (KLETT UNITS)
200
400
0
10
30
50
70
TIME (hrs)

inhibited 48%.

THE EFFECT OF NITRITE ON GLUCOSE METABOLISM BY RESTING CELLS

Table V shows the effect of nitrite on the glucose and nitrite utilization as well as on lactate production by resting cell suspensions under anaerobic conditions. Nitrite was supplied to the cell suspension at concentrations ranging from 50 to 200 umoles/50 ml; nitrate reduction by resting cells had previously produced nitrite concentrations ranging from 65 to 175 umoles/50 ml under anaerobic conditions. Nitrite had little or no effect on the total amounts of glucose utilized. Lactate production was markedly inhibited by nitrite. The small amounts of nitrite which were metabolized were proportional to the amount of nitrite supplied. The effect of nitrite on the glucose metabolism by resting cell suspensions under aerobic conditions was not tested.

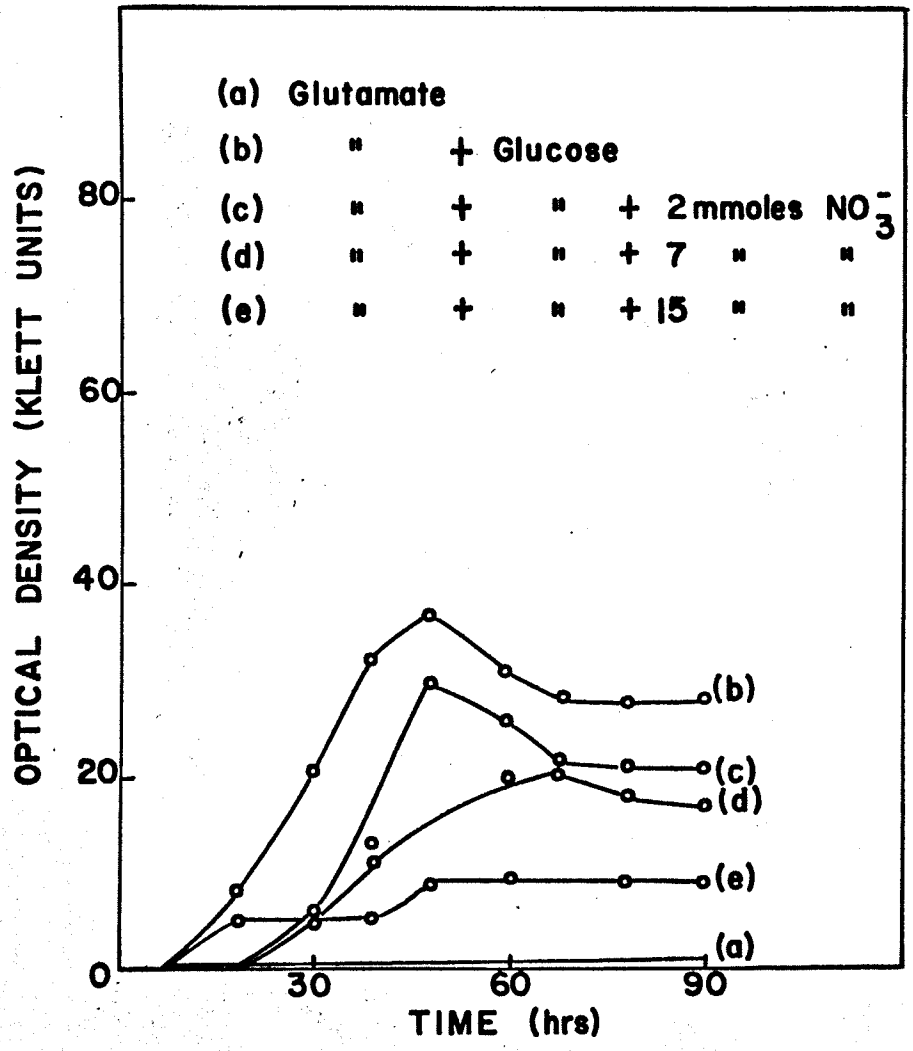
EFFECT OF NITRATE ON GLUCOSE METABOLISM IN GLUTAMATE-SUBSTITUTED MEDIUM

Figure 6a shows the effect of nitrate on anaerobic growth in a medium in which ammonia, the nitrogen source supplied as ammonium sulfate, was replaced by an equimolar amount of D,L-glutamic acid and K_2SO_4. Nitrate in the presence of glutamate still exerted an inhibitory

TABLE V. The effect of nitrite on glucose metabolism by resting cell suspensions of *E. coli* under anaerobic conditions.

NO_2^- supplied (μmoles/50 ml)	Glucose supplied (μmoles)	Glucose utilized (μmoles)	Lactate prod. (μmoles per μmole glucose utilized)	NO_2^- utilized (μmoles per μmole glucose utilized)
0	200	178	1.35	--
50	200	181.5	0.07	0.26
100	200	184.5	0.06	0.47
150	200	184.3	< 0.01	0.67
200	200	184.3	< 0.01	0.86

FIGURE 6a. Effect of nitrate on growth of E.coli strain B under anaerobic conditions with glutamate as the source of assimilatory nitrogen replacing $(NH_4)_2SO_4$ in the medium. Concentrations are expressed as mmoles of NO_3^- supplied as KNO_3 to the medium.


(a) Glutamate
(b) " + Glucose
(c) " + " + 2 mmoles NO_3^-
(d) " + " + 7 " "
(e) " + " + 15 " "
OPTICAL DENSITY (KLETT UNITS)
0
20
40
60
80
30
60
90
TIME (hrs)
(b)
(c)
(d)
(e)
(a)

effect. Figure 6a also shows that glutamate could not act as the sole carbon source for anaerobic growth. Ammonia proved to be a better nitrogen source than did glutamate (see Fig.5a).

Figure 6b shows the effect of nitrate on aerobic growth in the glutamate-substituted medium. Glutamate increased the length of the lag phase of growth five-fold. Nitrate in the presence of glutamate still exerted an inhibitory effect. Again, glutamate failed to serve as the sole carbon source for E. coli under aerobic conditions. Ammonia again proved to be a better nitrogen source than did glutamate (see Fig.4b).

MANOMETRIC EXPERIMENTS

Figure 7 shows the effect of various cell concentrations on oxygen uptake by E. coli with glucose as the oxidizable substrate. A wet weight of 34 mgm of cells showed no increased rate of oxygen uptake over 24.5 mgm of cells suggesting that at this cell density range an oxygen deficiency might be operative. To avoid this possibility 17 mgm wet weight (corresponding to 0.5 ml of the standardized cell suspension) were routinely used in all manometric studies.

The effect of rotenone on glucose metabolism by resting cell suspensions of E.coli was measured. The data of Figure 8 shows that 7×10^{-5}M rotenone did not impair glucose

FIGURE 6b. Effect of nitrate on growth of *E.coli* strain B under aerobic conditions with glutamate as the source of assimilatory nitrogen replacing $(NH_4)_2SO_4$ in the medium. Concentrations are expressed as mmoles as KNO_3 to the medium.

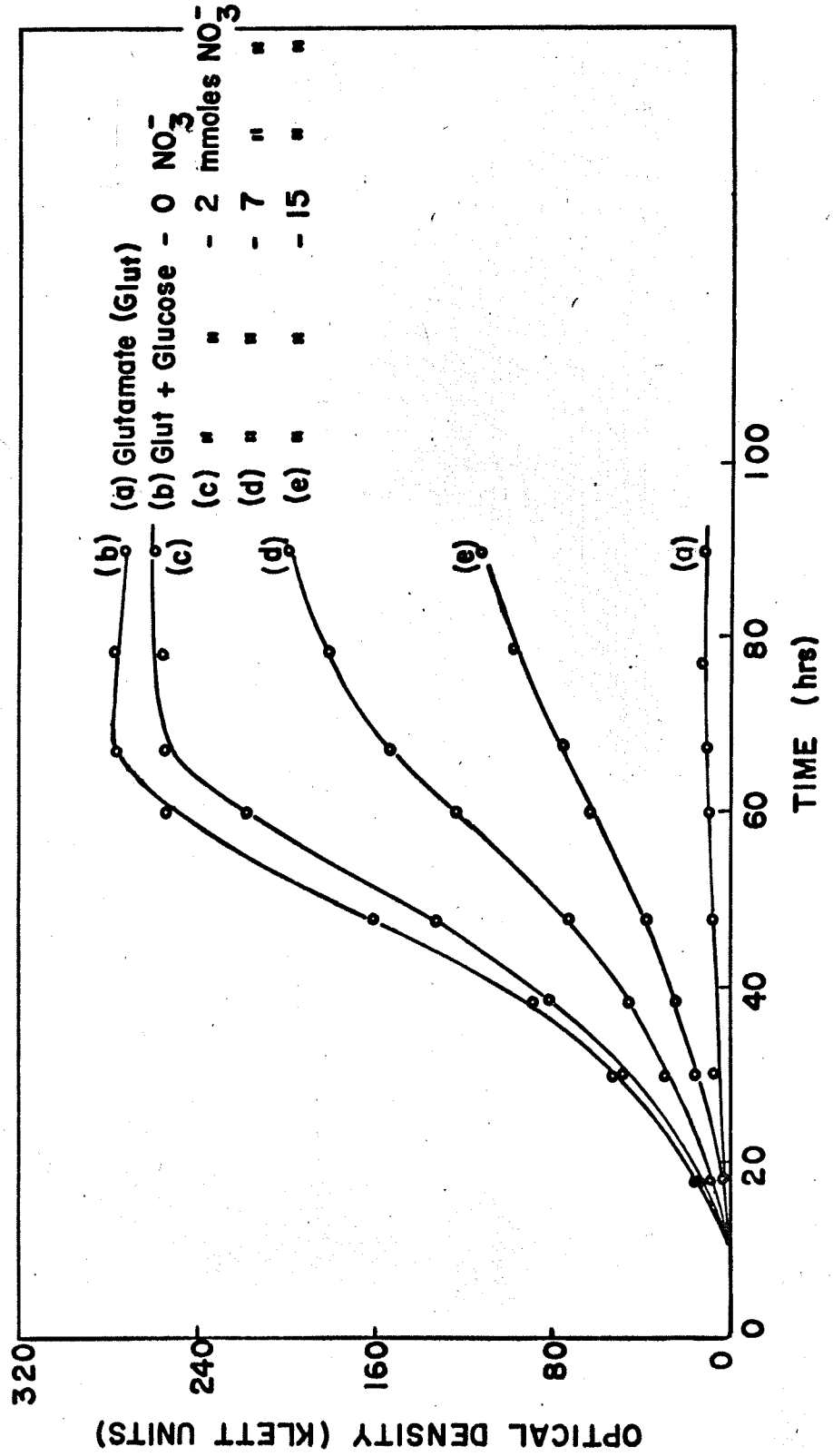

(a) Glutamate (Glut)
(b) Glut + Glucose - 0 NO_3^-
(c) " " - 2 mmoles NO_3^-
(d) " " - 7 " "
(e) " " - 15 " "
(a)
(b)
(c)
(d)
(e)
OPTICAL DENSITY (KLETT UNITS)
0
80
160
240
320
0
20
40
60
80
100
TIME (hrs)

FIGURE 7. Effect of various cell concentrations on the glucose oxidation of resting cell suspensions of E.coli strain B. Oxygen uptake was measured in µl and curves are endogenous plots representative of several trials. Concentrations of cells are expressed as mgm of wet weight.

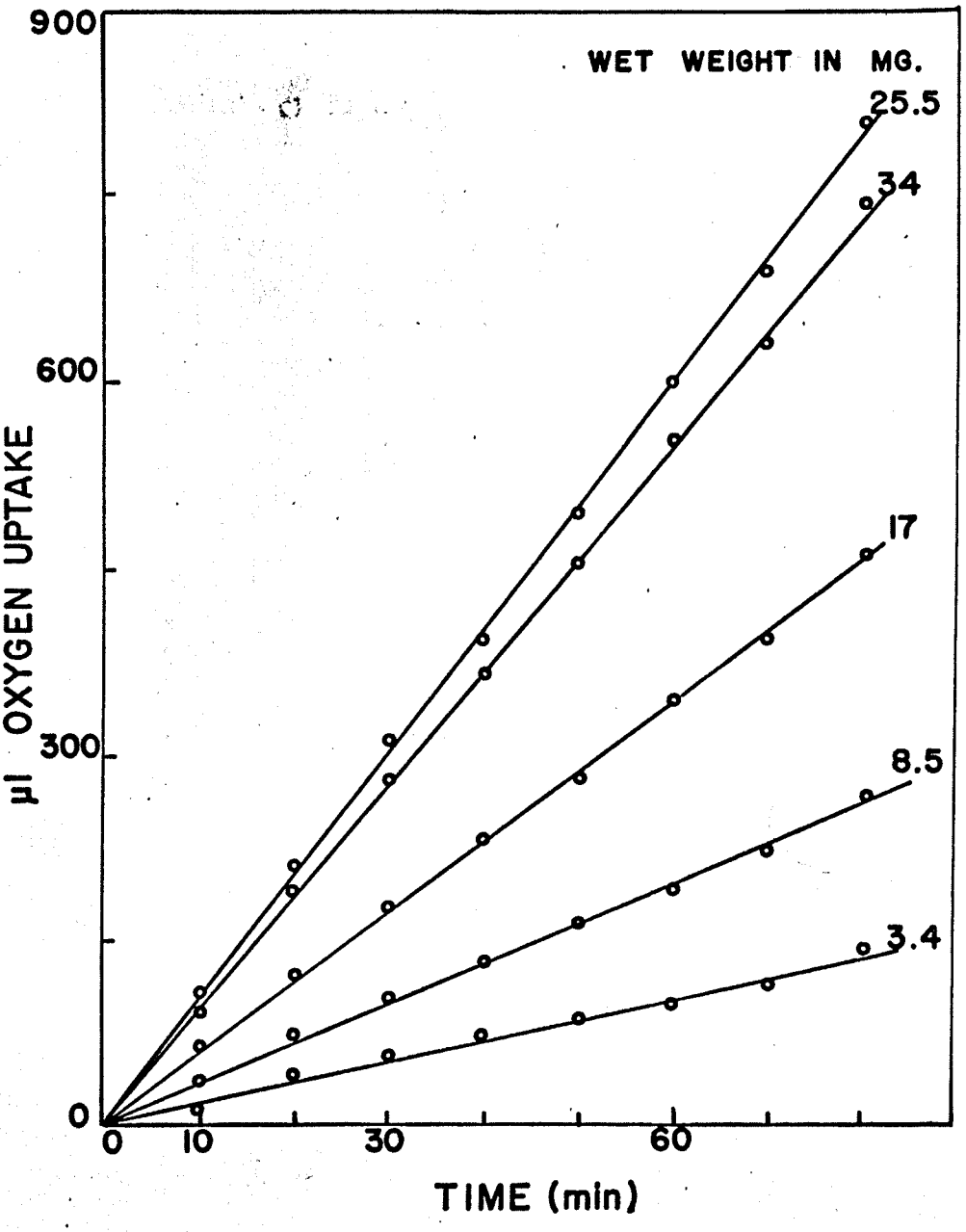
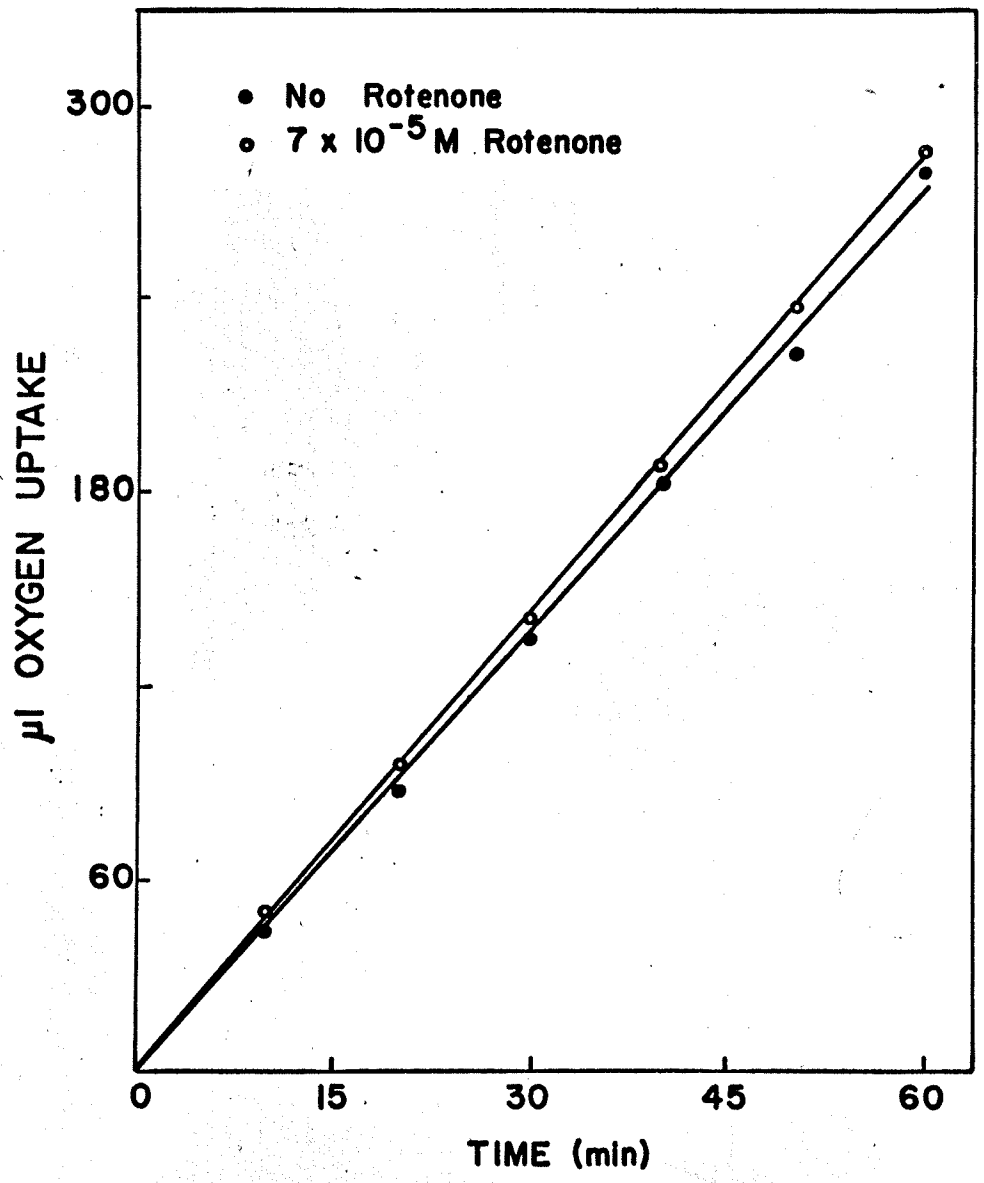

900
600
300
0
µl OXYGEN UPTAKE
WET WEIGHT IN MG.
25.5
34
17
8.5
3.4
0
10
30
60
TIME (min)

FIGURE 8. Effect of rotenone on the oxidation of glucose by resting cell suspensions of *E.coli* strain B. Oxygen uptake was measured in µl and curves are exogenous plots representative of several trials.


300
180
60
μl OXYGEN UPTAKE
• No Rotenone
o 7 x 10⁻⁵ M Rotenone
0
15
30
45
60
TIME (min)

oxidation. This concentration of rotenone was the highest that could be achieved due to the limited solubility of rotenone.

Figure 9 shows the effect of 10^{-3} and 10^{-4} M EDTA (in the presence and absence of rotenone) on glucose oxidation by resting cell suspensions. EDTA did not decrease oxygen uptake in the presence of rotenone. No significant physiological effect on glucose oxidation could be attributed to these concentrations of EDTA.

Rotenone did not impair glucose oxidation even when the resting cell suspensions were pre-treated with Tween 80 at concentrations of 0.36% and 0.014% prior to use. These results are shown in Figure 10. Oxygen uptake was not significantly affected by the pre-treatment procedure.

FIGURE 9. Effect of rotenone on the oxidation of glucose by resting cell suspension of *E. coli* strain B. in the presence of 10^{-3}M EDTA. Oxygen uptake was measured in µl and curves are exogenous plots representative of several trials.

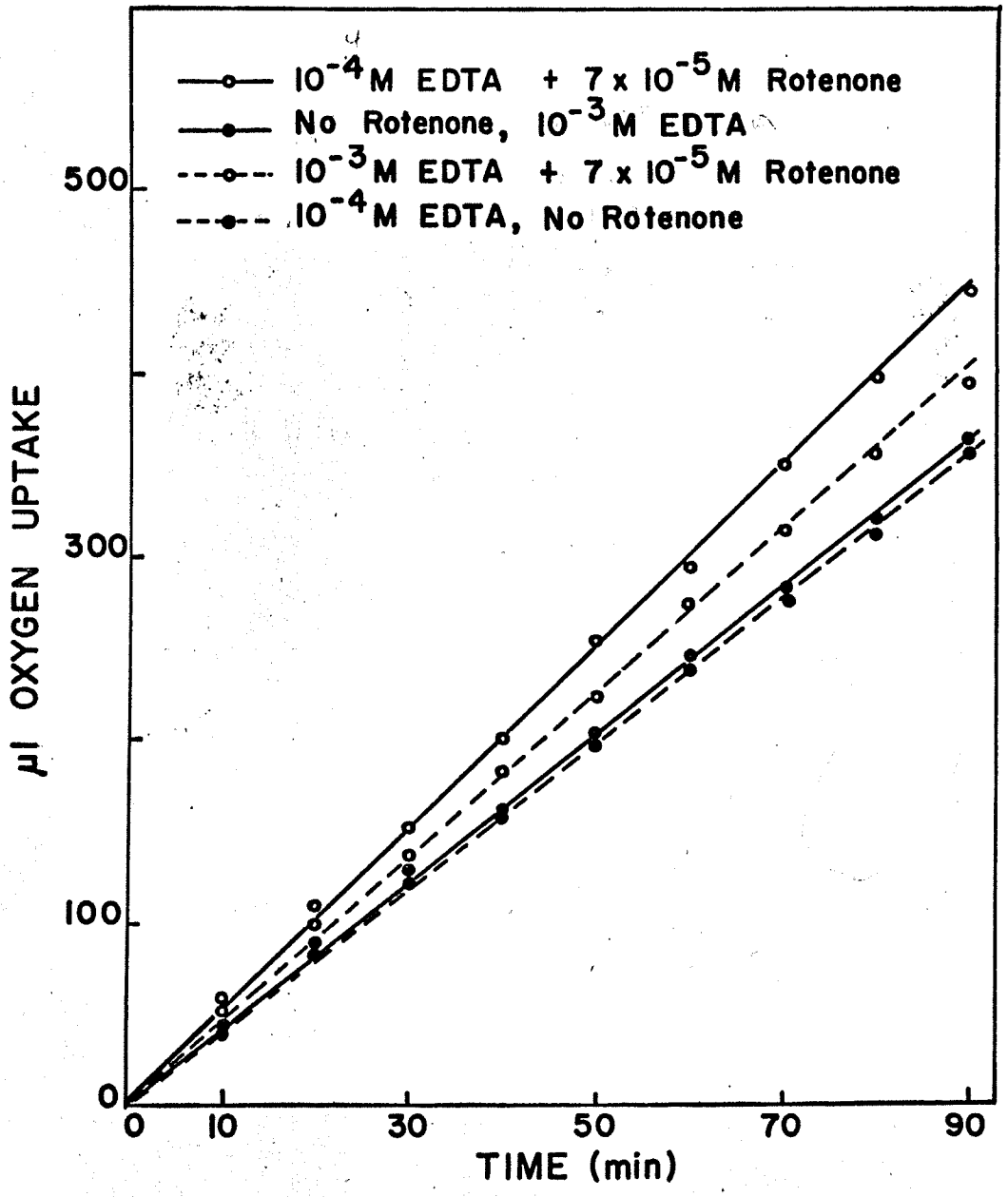
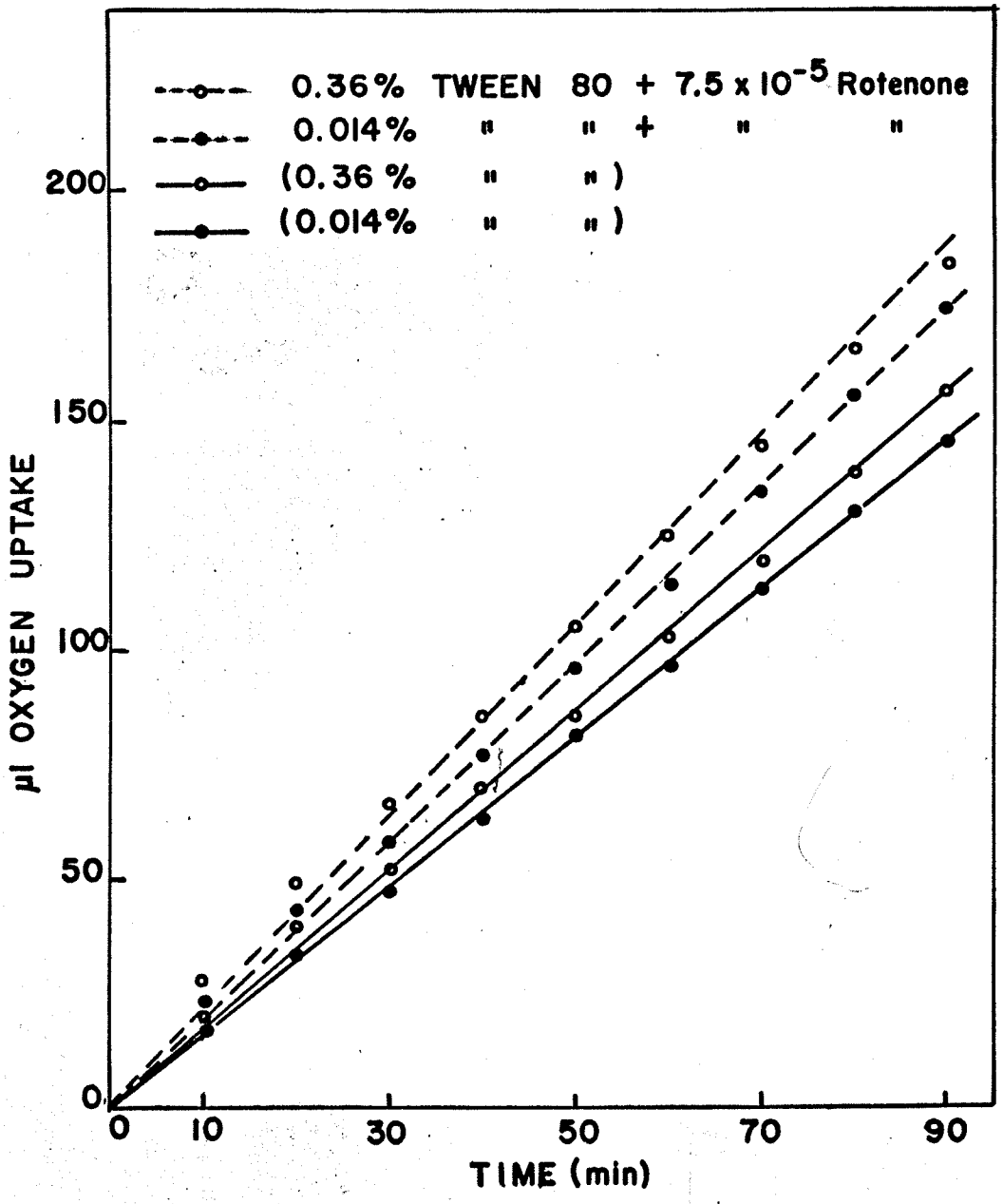

10⁻⁴M EDTA + 7 x 10⁻⁵M Rotenone
No Rotenone, 10⁻³M EDTA
10⁻³M EDTA + 7 x 10⁻⁵M Rotenone
10⁻⁴M EDTA, No Rotenone
μl OXYGEN UPTAKE
500
300
100
0
0
10
30
50
70
90
TIME (min)

FIGURE 10. Effect of rotenone on the oxidation of glucose by resting cell suspensions of *E. coli* strain B treated with 0.4 and 0.016% Tween 80. Oxygen uptake was measured in µl and curves are exogenous plots representative of several trials.


0.36% TWEEN 80 + 7.5 x 10⁻⁵ Rotenone
0.014% " " + " "
(0.36 % " ")
(0.014% " ")
200
150
100
50
0
µl OXYGEN UPTAKE
0
10
30
50
70
90
TIME (min)

DISCUSSION

DISCUSSION

Studies on resting and growing cultures of Escherichia coli strain B have shown that a Pasteur effect is operative in this organism. Levels of growth and CO_2 production were both lower in anaerobic cultures as compared to aerobic cultures. Anaerobic lactate production was higher than aerobic lactate production. These results were expected in an organism exhibiting a Pasteur effect. The results from these three parameters are sufficient to establish the existence of the Pasteur effect in this organism.

A more positive demonstration of the Pasteur effect would have been attained had rotenone successfully inhibited oxygen uptake by cell suspensions as described by Cereijo-Santalo and Wenner (8). Under the conditions employed in the course of this study however rotenone failed to inhibit oxygen uptake. This may have been because the cytochrome system of this organism is not sensitive to rotenone, or because of a failure of rotenone to penetrate the cell membrane. Preliminary attempts to increase the permeability of the cell membrane were restricted to mild treatments. The cytochrome system attached to the cell membrane is very labile. Tween 80 and EDTA thought to increase the permeability of the cell membrane, did not affect the activity of rotenone on cell suspensions of E. coli strain B. Clearly this area requires further study.

Nitrate has inhibited the final cell turbidity of both aerobically and anaerobically grown cells. Regardless of the presence of oxygen, whenever lactate and CO_2 (produced by both growing and resting cell suspensions) were present in detectable amounts they were found to be inhibited by nitrate. Lactate was the major detectable end-product of anaerobic glucose catabolism while CO_2 was the major detectable end-product of aerobic glucose catabolism. In only one case did the presence of nitrate affect the amount of glucose utilized. With resting cell suspensions under aerobic conditions the glucose utilized was slightly stimulated by the presence of nitrate; this may be an artifact and should be confirmed. Nitrate reduced the percentage of carbon recovered as lactate and CO_2 with both aerobically as well as anaerobically growing cells. Experiments with resting cells showed the same results. It seems that nitrate has altered the end-product patterns of glucose catabolism. This study was concerned with the effect of nitrate on growth and on CO_2 and lactate production in order to determine if nitrate will effectively replace oxygen as the terminal electron acceptor. The fate of the glucose-carbon in the presence of nitrate was considered to be beyond the scope of this study. The direction of carbon flow in the presence of nitrate however is an area which could bear further study.

The data suggest that nitrate has been reduced stoichiometrically to nitrite both by growing and resting cell suspensions. This is especially true of cells under anaerobic conditions which reduce much greater amounts of nitrate. This agrees with Nason (27) who has stated that the respiratory nitrate reductase is induced by anaerobic conditions. It was demonstrated that nitrite was inhibitory to both anaerobic growth and lactate production. The nitrite concentrations which inhibited growth and lactate production were in the same range as the nitrite concentrations produced from nitrate reduction. Because Lazzarini and Atkinson (21) have demonstrated the presence of a nitrite reductase in E. coli, it was speculated that the ammonia supplied in the medium might have inhibited the nitrite reductase and caused an accumulation of the inhibitory nitrite ion. Attempts were made to replace $(NH_4)2SO_4$ with glutamate as the source of assimilatory nitrogen. However, since much lower levels of growth were obtained both aerobically and anaerobically, ammonia proved to be the better nitrogen source.

The amount of nitrate metabolized both by resting and growing cell suspensions was approximately the same as the amount of nitrite produced. This suggests that the nitrite was not reduced further. Resting cell suspensions under anaerobic conditions were able to utilize a portion of the nitrite supplied. No attempt was made to determine the

nature of the products of nitrite reduction.

According to the scheme of Ota (31), only 1 mole of ATP is produced from the oxidation of each mole of $NADH_2$ by nitrate. Oxygen produces 3 moles of ATP in the oxidation of each mole of $NADH_2$. As a result, nitrate was not expected to attain the efficiency of oxygen as a terminal electron acceptor. Nitrate however was expected to replace oxygen at a reduced efficiency; it failed to do so.

An increase in anaerobic growth levels and CO_2 production as well as a decrease in lactate production was expected if nitrate acted effectively in replacing oxygen as a terminal electron acceptor in this organism. The fact that nitrate and its reduction product nitrite both inhibited anaerobic growth and CO_2 production shows that oxygen cannot be effectively replaced by nitrate.

The decrease in anaerobic lactate production in the presence of nitrate however suggests that nitrate might successfully have replaced oxygen as the terminal electron acceptor. The results show that the presence of nitrate inhibited all parameters measured. The anaerobic lactate inhibition caused by the presence of nitrate could have been a continuation of this general inhibition. It also seems logical that nitrate reduction and lactate production were in competition for $NADH_2$. If so, the utilization of $NADH_2$ by nitrate could account for nitrate inhibition of

anaerobic lactate production. It would appear then that nitrate has not been successful in replacing oxygen as a terminal electron acceptor and thus alleviating the Pasteur effect, perhaps because of an inhibition of growth due to the accumulation of the toxic nitrite ion.

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